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A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse *slaty* locus

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We have cloned and sequenced mouse cDNAs corresponding to a third member of a family of melanocyte-specific mRNAs, which encode tyrosinase and related proteins. This new member, tyrosinase-related protein-2 (TRP-2), has ~40% amino acid identity with the two other proteins in the family and has the same structural features including two copper binding sites, two cysteine-rich regions, a signal peptide and a transmembrane domain. We now show that one of the cysteine-rich regions in this protein family is an 'EGF-like' repeat found in many extracellular and cell surface proteins. The gene encoding TRP-2 maps to mouse chromosome 14, in the region of the coat colour mutation *slaty*. We show that the TRP-2 of *slaty* mice has a single amino acid difference from wild-type TRP-2; a substitution of glutamine for arginine in the first copper binding site. TRP-2 is the much sought melanogenic enzyme DOPAchrome tautomerase (DT), which catalyses the conversion of DOPAchrome to 5,6,dihydroxyindole-2-carboxylic acid. Extracts from mice homozygous for the *slaty* mutation have a 3-fold or more reduction in DT activity, indicating that TRP-2/DT is encoded at the *slaty* locus, and the missense mutation reduces but does not abolish the enzyme activity.

Key words: DOPAchrome tautomerase/melanocytes/mouse genetics/mutations/pigmentation

Introduction

Mutations at numerous loci affecting mouse hair colour have been invaluable in dissecting the developmental processes leading to pigment production by melanocytes (Silvers, 1979; Jackson, 1985, 1991). Several of these pigmentation genes have many mutant alleles, resulting from the specific locus mutation tests (Russell, 1951; Searle, 1974). Recombinant cDNAs have been cloned from three of these six specific loci, namely *dilute*, *albino* and *brown*. The first of these encodes a novel myosin heavy chain protein, involved in melanocyte shape and pigment distribution (Mercer *et al.*, 1991). The other two encode proteins related to each other. The *albino* locus on mouse chromosome 7 encodes the enzyme tyrosinase (Muller *et al.*, 1988; Kwon *et al.*, 1989; Takeda *et al.*, 1989; Yamamoto *et al.*, 1989) a melanocyte-specific enzyme which converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA), DOPA to DOPAquinone and possibly also 5,6-dihydroxyindole to indole-5,6-quinone (Korner and Pawelek, 1982). Deletions or point mutations in the tyrosinase gene give rise to loss or reduction of enzyme activity, and thus to complete or partial loss of all pigment (Jackson and Bennett, 1990; Shibahara *et al.*, 1990; Yokoyama *et al.*, 1990). Mice homozygous for the *albino* mutation can be restored to wild-type phenotype by transgenic insertion of the wild-type tyrosinase gene (Tanaka *et al.*, 1990; Yokoyama *et al.*, 1990).

A protein with ~36% amino acid identity to tyrosinase, termed tyrosinase-related protein-1 (TRP-1), was initially isolated as a candidate for tyrosinase (Shibahara *et al.*, 1986). Subsequent mapping and functional studies showed that TRP-1 maps to the *brown* locus on chromosome 4 (Jackson, 1988; Muller *et al.*, 1988) whereas tyrosinase is encoded by the *albino* locus on chromosome 7. At the *brown* locus deletions or null point mutations do not eliminate pigmentation, but result in mice with brown, instead of black eumelanin (Zdarsky *et al.*, 1990). Other mutations at this locus result in an intermediate phenotype, between black and brown, due to a reduction in the amount of TRP-1 mRNA, whilst still others have a dominant phenotype resulting in premature melanocyte death (Jackson *et al.*, 1990). Homozygous *brown* melanocytes in culture can be restored to wild-type pigmentation by the introduction of the wild-type TRP-1 sequence in a recombinant retrovirus (Bennett *et al.*, 1990).

A cDNA, termed 5A, was previously identified by screening of a melanocyte cDNA expression library with anti-tyrosinase antibodies (Jackson, 1988). The clone shows some cross-hybridization with pMT4, a TRP-1 cDNA clone, but encodes a different protein, which we term TRP-2 (tyrosinase-related protein-2). We report here the sequence of TRP-2, which is homologous to TRP-1 and tyrosinase and retains many of the features common to these two proteins. Antibodies to the C-terminal portion of TRP-2 detect a protein in melanocytes and melanoma of both mouse and humans. The protein has DOPAchrome tautomerase (DT) activity (see Discussion and Tsukamoto *et al.*, 1992). Genetic mapping using an interspecific backcross indicates that the gene is unlinked to either previously described locus, and is located distally on chromosome 14 at or near to a pigmentation locus called *slaty* (*slt*). The sequence of TRP-2 from *slaty* mice shows a single base (and single amino acid) difference from wild-type, located within one of the putative copper binding sites of the protein. The change results in a 3- to 4-fold decrease in DT activity, and a consequent change in the pigmentation of the mutant mice to a dark grey/brown eumelanin.

Results

Isolation and sequencing of TRP-2 cDNA

Previously we have screened a B16 mouse melanoma cell cDNA library cloned into the expression vector λgt11 with two rabbit antisera; one raised against mouse tyrosinase, the other against hamster tyrosinase. A clone (5A) was identified which was detected by both sera (Jackson, 1988). This clone hybridized at low stringency to the TRP-1 cDNA clone pMT4. However, 5A detected a different set of DNA fragments on Southern blot hybridization, and a slightly different sized melanocyte-specific mRNA species on Northern blot hybridization, and thus represents a different but related cDNA which we call TRP-2. We determined the DNA sequence of the complete 1180 bp cloned insert, which is a partial-length cDNA. Using a deletion subclone containing the 5′-terminal 330 bp we rescreened the cDNA library by hybridization. Five strongly positive clones were analysed. Three contain the same 1700 bp *Eco*RI fragment, but also contain an additional, smaller fragment. The clone containing the longest additional fragment (450 bp) was selected and both *Eco*RI fragments subcloned. We determined the DNA sequence of these fragments, by making and sequencing nested deletion derivatives. Subsequently we synthesized oligonucleotides which enabled sequence priming throughout the length of the coding region, which were used to confirm the sequence (see Materials and methods). The complete sequence shown in Figure 1 is compiled from the sequence of both cDNA clones, and confirmed by direct sequencing of PCR-amplified cDNA. (Each cDNA clone contained a single base pair variant from the authentic wild-type sequence determined from the PCR products.)

Also in Figure 1 is the translation of the amino acid sequence of TRP-2. The ATG initiation codon shown is the N-terminal end of the longest open reading frame. Within the first 200 bp of the 5′-untranslated sequence there are five ATG triplets, all of which are followed within 40 bp by an in-frame termination codon. Furthermore, comparison of the predicted protein sequence with translation of tyrosinase and TRP-1 mRNAs suggests that the N-terminus of the primary translation product of TRP-2 should be at the position indicated. Tyrosinase is known to be a membrane-inserted protein. Like tyrosinase and TRP-1, TRP-2 has both a N-terminal leader sequence and a potential transmembrane domain near the C-terminus, both shown in Figure 1. The '-1,-3' rule of von Heijne (1986) predicts that cleavage of the signal sequence should take place between the alanine and glutamine residues where indicated. This position is equivalent to the putative signal peptide cleavage sites of tyrosinase and TRP-1. There are 15 cysteine residues in conserved positions between tyrosinase, TRP-1 and TRP-2. These are circled in Figure 1. In addition TRP-2 has seven putative glycosylation sites of the form NXS or NXT. Of these, three are in conserved positions in both other family members and the other four are found in either tyrosinase or TRP-1. The C-terminal peptide used to raise antibodies is boxed.

Mapping of Tyrp-2 gene

In accord with mouse nomenclature rules we have named the gene encoding TRP-2, *Tyrp-2*. We chromosomally localized *Tyrp-2*, by analysing interspecific backcross progeny derived from the matings of [(C57BL/6J×*M.spretus*)F_1×C57BL/6J] mice. This interspecific backcross mapping panel has been typed for well over 800 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M.spretus* DNAs were digested with several enzymes and analysed by Southern blot hybridization for informative restriction fragment length variants using the *Tyrp-2* probe. The 7.6, 4.1 and 2.0 kb *M.spretus*-specific *Pvu*II fragments (see Materials and methods) were used to follow the segregation of the *Tyrp-2* locus in backcross mice. The mapping results indicated that *Tyrp-2* is located in the distal region of mouse chromosome 14. The other markers included in this study are *Bmp-1*, *hr* and *D14Ehl1*. Although 155 mice were analysed for every marker and are shown in the segregation analysis (Figure 2), up to 192 mice were typed for some markers. Each locus was analysed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice analysed for each pair of loci and the most likely gene order is:

centromere−*Bmp-1*−1/166−*hr*−10/192−*D14Ehl1*−39/190−*Tyrp-2*.

The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are: *Bmp-1*−0.6 ± 0.6−*hr*−5.2 ± 1.6−*D14Ehl1*−20.0 ± 2.9−*Tyrp-2*.

We have compared our interspecific mouse backcross map of chromosome 14 with the composite mouse linkage map compiled by M.T.Davisson, T.H.Roderick, A.L.Hillyard and D.P.Doolittle and provided from GBASE, a computerized database maintained at the The Jackson Laboratory, Bar Harbor, Maine. The region of chromosome 14 20−25 cM distal to *hr* contains three pigmentation genes. One, *s*, is a recessive spotting mutation, defective in melanocyte migration. Another, *Rn*, is a semi-dominant mutation which results in non- or partially pigmented hairs. The third is *slaty*, a recessive mutation which uniformly affects the eumelanin, so that on a *non-agouti* background, (in which the hairs are pigmented only with eumelanin) the mice are a dark grey/brown colour rather than black . In view of the brown phenotype resulting from mutations of TRP-1, we took *slaty* to be the best candidate for a mutation of TRP-2.

Expression and sequence of the slaty TRP-2 mRNA

The *slaty* mutation arose spontaneously on a non-inbred background. We obtained mice from The Jackson Laboratory in which the mutation had been backcrossed onto the C57BL/6J background. We prepared RNA from the skin of 5 day old homozygous *slaty* mice. Northern blot hybridization, probed with the TRP-2 cDNA shows that the amount of TRP-2 mRNA in the *slaty* mice is the same as in the wild-type C57BL/6 (Figure 3a).

We therefore used PCR to amplify the whole coding region of the TRP-2 cDNA made from the skin RNA, and purified the 1700 bp fragment by gel electrophoresis. Pairs of primers were used in PCR on the fragment to amplify overlapping 400−500 bp fragments of the cDNA which were sequenced directly with the priming oligonucleotides and with internal primers (see Materials and methods).

A single base pair difference only was found between the *slaty* TRP-2 coding region and the wild-type sequence in Figure 1. This is a G to A transition at base 985 which results

in a change in codon 194, changing the wild-type arginine residue to glutamine. Figure 3b shows the sequence in the region of this change. The transition has occurred at a G in the dinucleotide CpG. This dinucleotide is the common site of methylation of mammalian DNA and spontaneous mutations very frequently occur at these sites, most likely

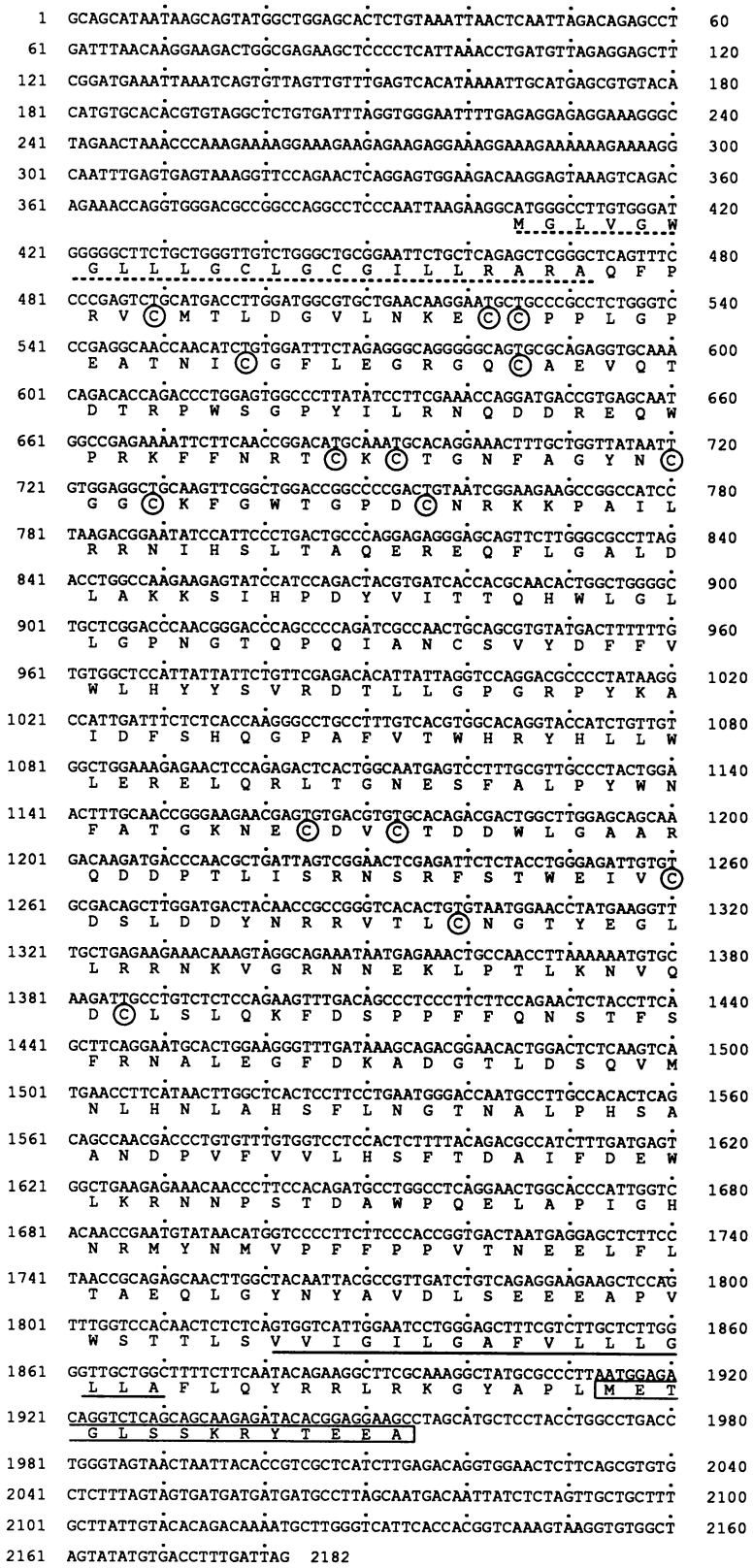

Fig. 1. Sequence of TRP-2 cDNA. The presumed full-length open reading frame is shown below the DNA sequence. All 15 cysteine residues (enclosed by circles) are conserved across the whole tyrosinase-related family. The N-terminal signal sequence is underlined with a dotted line, and the hydrophobic transmembrane domain has a solid underline. The boxed C-terminal 14 residue peptide (PEP8) was used to raise anti-TRP-2 antiserum. These sequence data are available from EMBL/GenBank/DDBJ under accession number X63349.

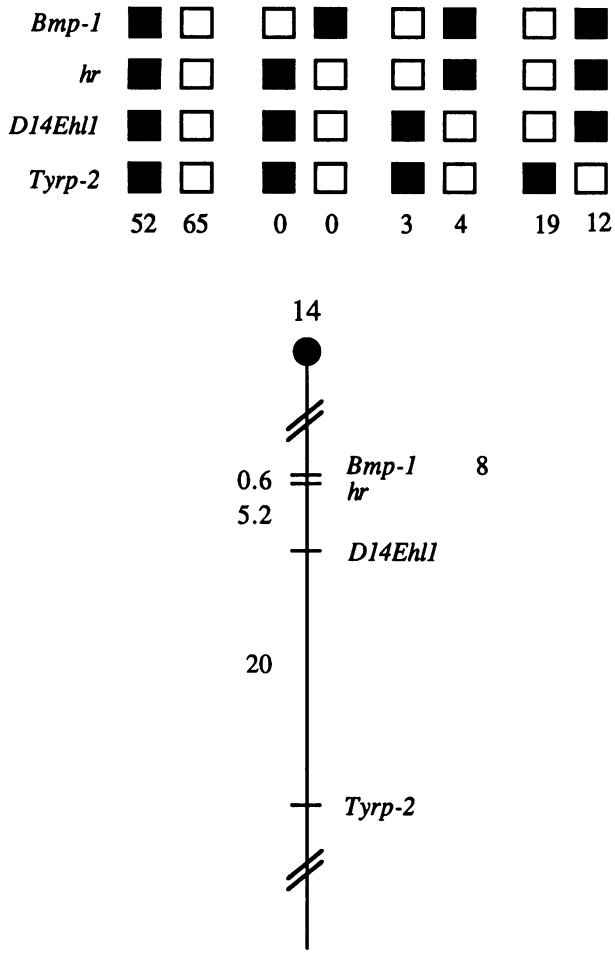

Fig. 2. Position of the *Tyrp-2* locus on mouse chromosome 14. *Tyrp-2* was placed on chromosome 14 by interspecific backcross analysis. The segregation patterns of *Tyrp-2* and flanking genes in 155 backcross animals that were typed in common for *Tyrp-2* is shown at the top of the figure. For each individual pair of loci, more than 155 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J×*M.spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a *M.spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Gene order between *Bmp-1* and *hr* was determined by the analysis of additional mice, not typed for *D14Ehl1* and *Tyrp-2*. These studies have been reported (Ceci *et al.*, 1990). A partial chromosome 14 linkage map showing the location of *Tyrp-2* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in cM are shown to the left of the chromosome and the position of *Bmp-1* on human chromosomes is shown to the right. Human chromosomal assignments for the other loci have not been obtained.

by the deamination of the methyl-C resulting in a T:G pair which, on replication can result in the substitution of G by A. The mutation destroys a *Taq*I restriction site. To show that the mutation is present in *slaty* cDNA we used PCR to amplify a 320 bp segment of cDNA from *slaty* skin, from two wild-type skin cDNAs (C57BL/6 and BALB/c) and from the B16 melanoma cell line from which the TRP-2 cDNA was initially cloned. Figure 3c shows that the *Taq*I site is present in the wild-type strain cDNAs but is absent from the TRP-2 transcript of *slaty* mice.

Expression and function of wild-type and slaty TRP-2

Antibodies were raised in rabbits against a peptide (PEP8) consisting of the C-terminal 14 amino acids of TRP-2

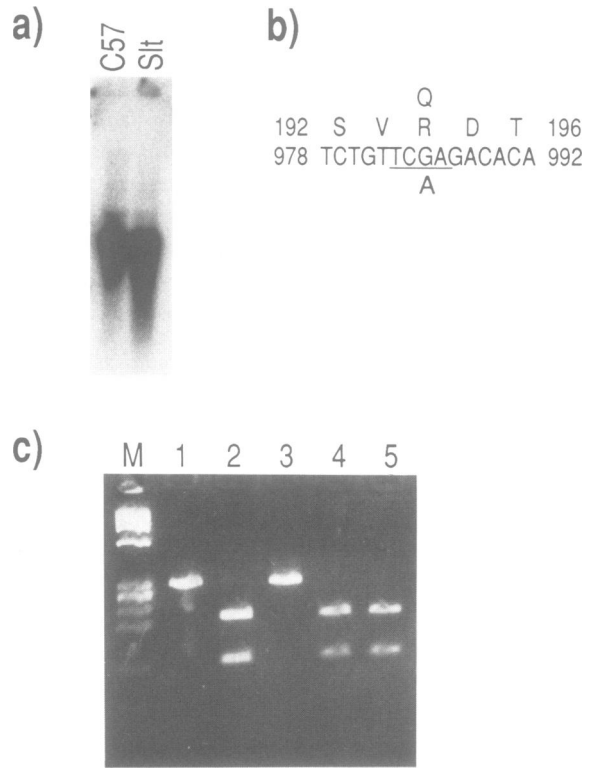

Fig. 3. The *slaty* mutant TRP-2. (**a**) Northern blot hybridization analysis of total skin RNA from neonatal C57BL/6J (left) and homozygous *slaty* (right) neonatal mice, probed with labelled TRP-2 cDNA. Equal amounts (10 μg) of RNA were loaded in each lane, and equal loading confirmed by ethidium bromide staining of a duplicate gel. (**b**) Sequence of cDNA and predicted protein in the region of the *slaty* mutation. DNA sequence shown is from bases 978 to 992, and the amino acids are from 192 to 196. The *slaty* mutant base (base 985 G to A) is below the sequence, and the resulting amino acid change (residue 194 R to Q) is above. The *Taq*I site which is lost in the *slaty* sequence is underlined. (**c**) PCR analysis of cDNA from total neonatal skin RNA. Lane 1, undigested PCR product; lane 2, *Taq*I-digested B16 melanoma cDNA; lane 3, *Taq*I-digested homozygous *slaty* neonatal skin cDNA; lane 4, *Taq*I-digested C57BL/6J neonatal skin cDNA; lane 5, *Taq*I-digested BALB/c neonatal skin cDNA.

coupled to BSA (Figure 1 and Tsukamoto *et al.*, 1992). The antiserum detects a 75 000 molecular weight glycoprotein in wild-type mouse melan-a melanocytes and B16 melanoma cells (Figure 4A). This size is larger than that predicted from the open reading frame in Figure 1, and is accounted for by glycosylation of the primary translation product, in common with tyrosinase and TRP-1. A 55 000 molecular weight precursor polypeptide can also be detected. The recognition of these peptides by anti-PEP8 sera confirms that the open reading frame as shown is expressed. These proteins are also detectable in human MNT1 melanoma cells (Figure 4A). A thorough characterization of the αPEP8 serum is described in Tsukamoto *et al.* (1992)

Extracts from the eyes of neonatal wild-type (C57BL6/J), *albino* (BALB/c) and *slaty* (congenic with C57BL/6J) mice were examined by Western blotting using the αPEP8 antibody to detect TRP-2. Eyes contain the retinal epithelium layer, which is made up of melanocytes, and therefore contain the melanogenic enzymes. The amount of TRP-2 immunoreactive protein detected in *slaty* mice is highly variable, sometimes being undetectable and at other times

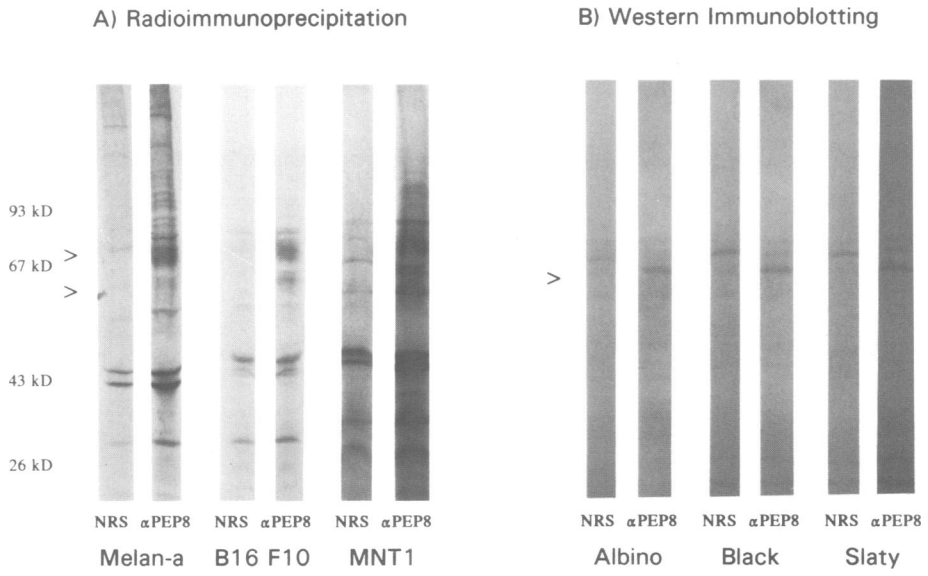

Fig. 4. Expression of TRP-2/DT protein. (**A**) Immunopreciptation of labelled peptides from wild-type mouse melanocytes (melan-a), wild-type mouse melanoma (B16 F10) and human melanoma (MNT 1) cells. Precipitations were carried out using normal rabbit serum (NRS) or antibodies specifically directed against the C-terminus of TRP-2 (αPEP8). The arrows indicate the unglycosylated precursor peptide of 55 kDa and the mature, glycosylated form of 75 kDa both seen in the αPEP8 lanes, but not the NRS. (**B**) Western blot detection of TRP-2/DT in eye extracts of *albino*, black (wild-type, C57BL/6J) and *slaty* (congenic with C57BL/6J) mice, reacted with normal rabbit serum (NRS) and with antibodies against the C-terminal peptide of TRP-2 (αPEP8).

being close to the amount seen in wild-type eyes. Figure 4B is a Western blot, in which TRP-2 is visualized in extracts from the three strains, showing that the protein detected in *slaty* mice is the same size as wild-type.

The protein precipitated by the αPEP8 serum has DOPAchrome tautomerase (DT) activity (Tsukamoto *et al.*, 1992). DT is an enzyme, previously known as DOPAchrome conversion factor, DOPAchrome oxidoreductase or DOPAchrome isomerase (Korner and Pawelek, 1980; Murray *et al.*, 1983; Barber *et al.*, 1984; Aroca *et al.*, 1990; Pawelek, 1990) which participates in melanin synthesis by converting DOPAchrome to 5,6,-dihydroxyindole carboxylic acid (DHICA). It has until now been only partially characterized. We propose that TRP-2 is DT, and will refer to it hereafter as TRP-2/DT.

Eye extracts from the same three strains examined in Figure 4B were assayed for enzyme activity. Table I summarizes the tyrosinase and DT activity from the three strains. The DT activity in *slaty* eyes is somewhat variable, but is consistently lower than in wild-type or *albino* extracts and the mean is less than one-third wild-type. Furthermore, the extraction of enzyme activity appears to be influenced by the melanin synthesized. The melanogenic enzymes are localized within the melanosomes, where melanin is synthesized and deposited. The enzymes become coated with melanin during its synthesis, and therefore more difficult to extract. This is seen as a higher yield of DT from albino eyes (without melanin) than black eyes (with wild-type melanin), and a higher recovery of tyrosinase from the *slaty* eyes compared with wild-type. The tyrosinase difference cannot be due to differences other than near the *slaty* locus as the *slaty* mice and wild-type are congenic. We may therefore take tyrosinase activity as a measure of enzyme extraction efficiency, and hence the relative activity of DT in *slaty* compared with wild-type falls to $<10\%$. This correlation between the point mutation in the TRP-2/DT protein in *slaty* mice and the decrease in DT activity in the same animals is a strong indication that the *slaty* locus does indeed encode TRP-2/DT.

Table I. Tyrosinase and DOPAchrome tautomerase activity in extracts of wild-type, *albino* and *slaty* tissues

Description	Tyrosinase (pmol/μg/h)	DT (pmol/μg/h)
Buffer control	0.0	0.0
slaty liver	0.03 ± 0.02 (n = 4)	0.00 ± 0.00 (n = 2)
Black eye	0.29 ± 0.06 (n = 6)	1.75 ± 0.65 (n = 4)
albino eye	0.01 ± 0.01 (n = 6)	2.25 ± 0.05 (n = 2)
slaty eye	0.97 ± 0.03 (n = 6)	0.50 ± 0.16 (n = 5)

Discussion

We have described here the identification of a third member of the tyrosinase protein family, TRP-2/DT. This appears to be an old family. The genes are dispersed and located on three different chromosomes, with no obvious chromosomal relationships. The overall amino acid identity between family members is of the order of 40%, which is scattered throughout the proteins. Figure 5 shows a comparison between the amino acid sequences of all three. Examination of silent base substitutions at codons for conserved amino acids suggests that the substitutions have reached saturation; that is, the genes have been evolving independently for a sufficiently long time that the only nucleic acid identity seen is that which is required for amino acid identity, and no relics remain of the ancestral gene. Further, when the gene structures of tyrosinase and TRP-1 are compared, their intron/exon organizations are quite different (Jackson *et al.*, 1991), again suggesting an ancient duplication. A clearer idea of the age of the gene triplication will be obtained by looking at the family in other species.

We examined the amino acid sequence of TRP-2/DT using the programme Prosearch (L.F.Kowalski, MIT) to compare

Fig. 5. Sequence comparison of tyrosinase-related protein family. The sequences of mouse tyrosinase (top row), TRP-2/DT (middle) and TRP-1 (lower) aligned to maximize amino acid similarity. Identical amino acids are indicated by | and amino acids with chemically similar properties by :. The 15 conserved cysteine residues are shaded, the two copper binding sites are boxed and the hydrophobic transmembrane domain is enclosed by brackets.

with the Prosite database of motifs of Bairoch (1991) and to carry out a sequence comparison with the protein sequence database using the distributed array processor. We have found that the first cysteine-rich domain is a previously unreported member of the 'EGF-like' family. This widespread motif consists of a number of cysteines, aromatic residues, and glycines in a characteristically spaced pattern (reviewed by Davis, 1990). Figure 6 shows the comparison between the TRP region (from mouse and human tyrosinase and TRP-1 in addition to TRP-2/DT) and consensuses of different members of the EGF family. Some of these have, like the TRPs, only a single related cysteine-rich region. These proteins include numerous growth factors, such as EGF itself, TGF-α and vaccinia growth factor. The blood

TRPs [x1]	C	X	–	C	X	X	X	X	X	G	F/Y	X	C	X	X	C	X	X	G	F/W	X	G	X	X	C
laminin B_1 [x13]	C	X	–	C	X	X	X	X	X	G	X	X	C	X	X	C	X	X	G	F/Y	X	G	X_{3-13}		C
laminin A [x20]	C	X_{1-2}		C	X	X	X	X	X	G	X	X	C	X	X	C	X	X	G	F/Y	X	G	X_{3-17}		C
laminin B_2 [x12]	C	X_{1-2}		C	X	X	X	X	X	G	X	X	C	X	X	C	X	X	G	F/Y	X	X	X_{5-11}		C
tenascin [x13]	C	X_5		C	–	–	–	X	X	G	–	X	C	X	–	C	X	X	G	F/Y	X	G	X	X	C
serrate [x13]	C	X_5		C	X	X	X	X	X	G	F/Y	X	C	X	–	C	X	X	G	F/Y	X	G	X	X	C
Notch [x36]	C	X_5		C	X	X	X	X	X	X	F/Y	X	C	X	–	C	X	X	G	F/Y	X	G	X	X	C
lin –12 [x13]	C	X_{5-9}		C	X	X	X	X	X	X	F/Y	X	C	X	–	C	X	X	G	F/Y	X	G	X	X	C
growth factors [x1]	C	X_{4-5}		C		X_8					F/Y	X	C	X	–	C	X	X	G	F/Y	X	G	X	X	C
clotting factors [x2]	C	X_5		C	X	X	X	X	X	X	F/Y	X	C	X	–	C	X	X	G	F	X	G	X	X	C

Fig. 6. Comparison of the tyrosinase-related protein EGF domain. Alignment of amino acid sequences of some of the proteins containing the EGF domain. The single-letter amino acid code is used, X = any amino acid residue, – = gap introduced to maximize alignment. The key conserved residues are enclosed by a box. References to all sequences can be found in Davis (1990), except serrate, which is Fleming *et al.* (1990) and Thomas *et al.* (1991).

clotting factors X and XI have two motifs, whilst others have many copies, over 30 in some cases.

The best match to the TRP EGF domain is with the multiple repeats in the extracellular matrix proteins, laminins A, B1 and B2. The spacing of the conserved cysteines, in addition to conserved glycine and aromatic residues is identical between the TRPs and the laminin repeats. Other extracellular proteins such as tenascin, fibulin and entactin have repeats in which some of the spacings are slightly different from the TRPs, but nevertheless are clearly related. Also related are a number of *Drosophila* developmental gene products, such as *Notch*, *Delta*, *crumbs*, *serrate* and *slit*, and the nematode developmental genes *lin-12* and *glp*.

The function of the EGF repeat is enigmatic. The pattern of disulphide bridges between cysteines, where it has been determined, is always 1–3, 2–4 and 5–6 (the first cysteine in Figure 6 is number 2). The two classic mouse mutations in tyrosinase (*albino*) and TRP-1 (*brown*) are substitutions of cysteines 5 and 4 respectively in this motif, and a recently characterized human albinism mutation has been found in cysteine 2 (Jackson and Bennett, 1990; Zdarsky *et al.*, 1990; Spritz *et al.*, 1991)

A common thread between the families which contain the EGF motif appears to be that all are extracellular proteins which may participate in protein–protein interaction. The TRPs are not extracellular, but rather are most likely bound to the inner (lumenal) face of the melanosome, the subcellular site of pigment synthesis. We propose that all three family members co-localize in the membrane, where they form a multi-enzyme complex which undertakes eumelanin synthesis. The EGF repeat is perhaps required for interaction between the proteins and the formation of the complex. In support of this notion Pawelek *et al.* (1991) have recently demonstrated that a high molecular weight complex can be isolated from melanosomes which contains tyrosinase, gp75 (human TRP-1) and DT (TRP-2). They have also demonstrated MSH-receptor function in this complex. It remains to be seen whether additional proteins participate.

The point mutation we identify is a strong candidate for the *slaty* mutation, and make it highly likely that TRP-2/DT is encoded at the *slaty* locus. Further genetic analysis of the locus is limited; the *slaty* mutation is the only mutant allele known. Nor is the background strain of origin of the mutation known. The arginine to glutamine mutation in the *slaty* TRP-2/DT is located in the first copper binding site of the protein. This amino acid residue is only moderately conserved between family members. The equivalent residue is also arginine in mouse TRP-1 and tyrosinase, and is the similarly basic amino acid lysine in human TRP-1, but in human tyrosinase the equivalent residue is methionine. To date, nine different missense mutations have been identified in human tyrosinase which produce type IA albinism (a complete loss of tyrosinase activity) (Giebel *et al.*, 1990; Kikuchi *et al.*, 1990; Spritz *et al.*, 1990, 1991; Takeda *et al.*, 1990; Oetting *et al.*, 1991a,b). All nine change amino acids which are identical across all five members of the tyrosinase-related family (two human and three mouse). In addition, the two loss-of-function cysteine mutations in mouse tyrosinase and TRP-1 are conserved across the whole family (Zdarsky *et al.*, 1990; Jackson and Bennett, 1990). By contrast, the two characterized mutations which lead to type IB (yellow) albinism, and which, like the TRP-2/DT *slaty* mutation do not give a complete loss-of-function, are in amino acid residues which, as in the *slaty* mutation, are only conserved across some of the family members (Giebel *et al.*, 1991).

Can the dark grey/brown phenotype of homozygous *slaty* mice be rationalized by the reduction of DT activity? In the absence of the enzyme DOPAchrome spontaneously converts to DHI. Ito (1986) has shown by acid degradation of melanins that melanins formed by tyrosinase alone *in vitro* contain ~10% DHICA, the rest being mostly DHI. By contrast, analysis of natural melanins, from melanoma cells or from black hair, consists of at least 50% DHICA. We have shown (Tsukamoto *et al.*, 1992) that little or no DHICA is made from DOPAchrome *in vitro* in the absence of DT, whether spontaneously or in the presence of tyrosinase alone. It seems likely that it is a difference in DHICA content which results in the different colours of wild-type (black) versus *slaty* eumelanin. The colour difference is a subtle one, which probably reflects the residual DT activity in *slaty* animals. It will be interesting to create null mutations of TRP-2/DT to observe their effects on pigmentation, and also to see if absence of DT activity has any other phenotypic effect on development. It is possible that complete lack of DT might have a severe effect on viability of melanocytes; DHI is quite a cytotoxic metabolite (Pawelek and Lerner, 1978; Pawelek *et al.*, 1980), while DHICA appears to be somewhat less toxic (unpublished).

In summary, we have identified a novel tyrosinase-related protein, shown that it maps to and is mutated at the coat colour locus, *slaty*, and shown that the mutant protein is deficient for DOPAchrome tautomerase activity. The identification of the mutant will be invaluable for further studies of the protein's function, and for further dissection of the development of melanocyte function.

Materials and methods

Isolation and sequencing of TRP-2 cDNAs

The identification of clone 5A as a λgt11 clone reacting with two different tyrosinase antisera has been described (Jackson, 1988). The 1.2 kb *Eco*RI insert was subcloned in both orientations into pBS. A series of deletion derivatives of the plasmids were made by exonuclease digestions from one end using the Erase-a-Base kit from Promega. These were sequenced using Sequenase (USB) according to the manufacturer's instructions. The 330 bp insert from a deletion plasmid, representing the 5'-most deletion product was used to rescreen the λgt11 library by DNA hybridization in order to identify full-length cDNA clones. The two *Eco*RI fragments of the largest clone were subcloned separately into pBS.

The longer fragment was reduced to deletion derivatives as above and sequenced. Oligonucleotide primers were synthesized which would prime sequencing along the whole length of the cDNA. These corresponded to bases in Figure 1 as follows:

coding strand complementary: 355–373; 543–562; 781–801; 1101–1120; 1306–1325; 1462–1483; 1642–1661; 1788–1807;

non-coding strand complementary: 565–546; 781–762; 1100–1081; 1305–1286; 1461–1442; 1641–1623; 1787–1769; 2025–2006.

These primers were used to confirm the sequence of the full-length clone.

The same primers were also used to prime the polymerase chain reaction (PCR) from cDNA synthesized using a kit from Boehringer-Mannheim and total RNA from neonatal wild-type or *slaty* mouse skin (Saiki *et al.*, 1988; Jackson *et al.*, 1990). A PCR was performed using the outermost primer pair. The 1671 bp fragment thus amplified was isolated from an agarose gel using Geneclean and was reamplified using primer pairs separated by 400–560 bp to generate overlapping amplified fragments. These were again gel-purifed and sequenced according to the method of Winship (1989) using the PCR-priming oligonucleotides and the internal pair.

RNA isolation and analysis

RNA was isolated from neonatal mouse skin as described (Jackson *et al.*, 1990) using the LiCl/urea method. Formaldehyde–agarose gel analysis was as described. The RNA was blotted onto nitrocellulose and hybridized with the 5A insert as described.

Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J×*M.spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 progeny were obtained; a random subset of these N_2 mice were used to map the *Tyrp-2* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The *Tyrp-2* probe, the 1.2 kb *Eco*RI fragment from the cDNA clone 5A, was labelled with [α-^{32}P]dCTP using a nick translation kit (Boehringer Mannheim). Fragments of 8.9, 4.8, 3.9 and 2.2 kb were detected in *Pvu*II-digested C57BL/6J DNA; fragments of 7.6, 4.1 and 2.0 kb were detected in *Pvu*II-digested *M.spretus* DNA. The 7.6, 4.1 and 2.0 kb *M.spretus*-specific fragments cosegregated and were followed in this analysis. A description of the probes and RFLPs for the bone morphogenetic protein-1 (*Bmp-1*) and hairless (*hr*) loci has been reported previously (Ceci *et al.*, 1990). The probe for DNA segment, chr 14 Ehl1 (*D14Ehl1*) locus was a 900 bp mouse cDNA that detected a 2.5 kb *Taq*I fragment in C57BL/6J DNA and a 4.2 kb fragment in *M.spretus* DNA. The probe was kindly provided by Elizabeth Lacy (Sloan-Kettering Institute, New York, NY). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Protein methods

Production of the PEP8 antibody is described in Tsukamoto *et al.* (1992). Melan-a melanocytes and B16 and MNT1 melanoma cells were grown as previously described. For metabolic labelling they were pulsed for 30 min with [^{35}S]methionine and chased for 60 min, harvested by trypsinization and solubilized in 1% Nonidet-P40, 0.01% SDS, 0.1 M Tris–HCl, pH 7.2, 1 μg/ml aprotinin and 100 mM PMSF as detailed in Tsukamoto *et al.* (1992). Immunoprecipitation with antibodies was as described, and reactive proteins were electrophoresed through 7.5% Laemmli polyacrylamide gels (Laemmli, 1970). and visualized by treatment with Autofluor (National Diagnostics, Manville NJ) and autoradiography on Kodak XAR-2 film.

Proteins were electroblotted onto nitrocellulose filters, reacted with 1:1000 dilutions of αPEP8 or non-immune serum and the antibodies detected with the avidin–biotin system coupled to alkaline phosphatase (Tsukamoto *et al.*, 1992).

Tyrosinase and DOPAchrome tautomerase activity was assayed in tissue extracts as described in Tsukamoto *et al.* (1992)

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